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14. ABSTRACT The threat of terrorist attacks involving radioactive material mandates the study and development of potential new treatments for bone marrow failure caused by acute radiation injury. A whole body dose of radiation even at levels as low as 1Gy can result in Bone Marrow Failure. Hematopoietic Acute Radiation Syndrome is characterized by bone marrow failure with life threatening neutropenia and thrombocytopenia. In addition to direct injury to hematopoietic cells, radiation is known to induce a pro-inflammatory cytokine storm in which various cell types produce cytokines, chemokines, growth factors and reactive oxygen species that can be especially detrimental to hematopoietic stem cells. Inflammation and oxidative damage induced by radiation exposure are significant contributors to bone marrow failure. We and our collaborators have recently shown that a short EPO-mimetic peptide improves survival in a murine model of acute radiation induced Bone Marrow Failure, however the peptide's molecular mechanism is unknown. The specific hypothesis behind the proposed research project is that the EPO-mimetic peptide decreases the mortality rate in a mouse model of radiation induced Bone Marrow Failure by directly inhibiting apoptosis in hematopoietic stem cells.					
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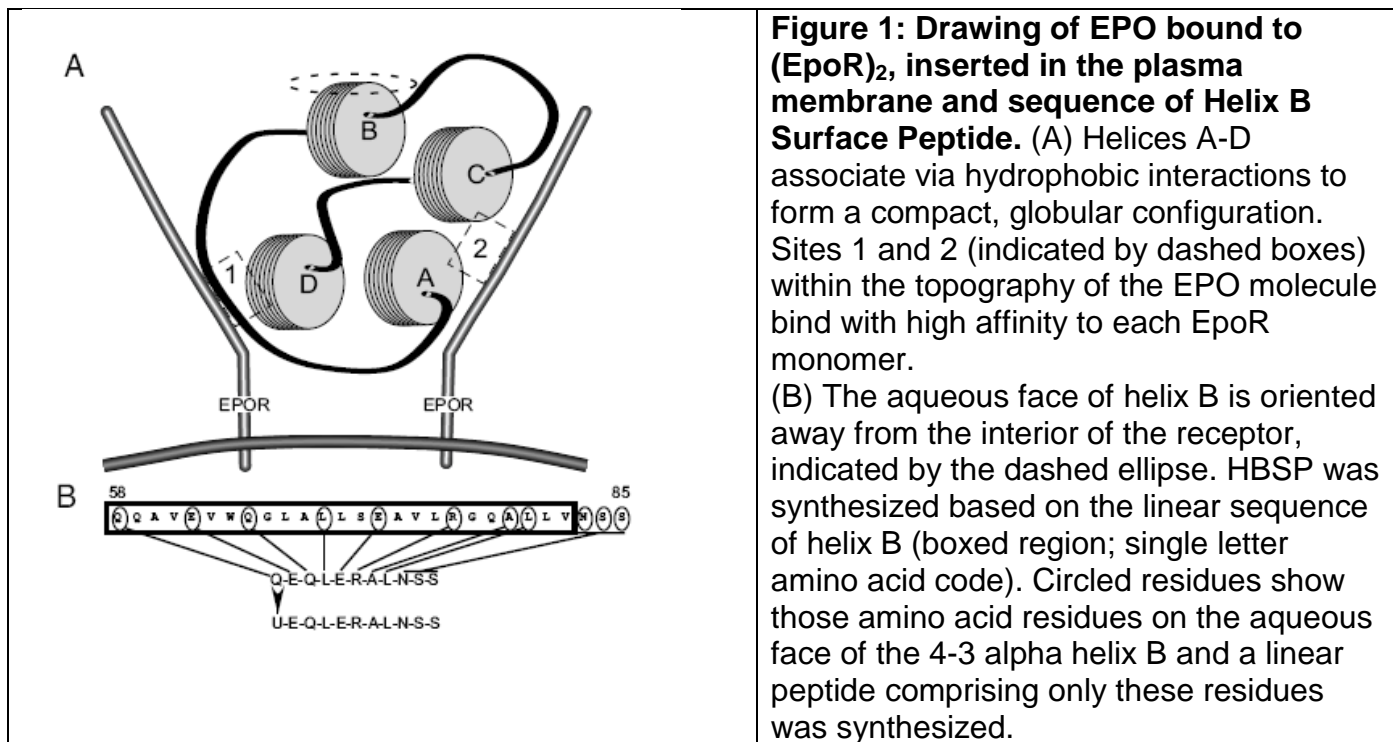
Introduction:

Simple, inexpensive and easily accessible therapies for bone marrow failure are currently lacking. Recently, the cytokine erythropoietin (EPO) has been shown to play an important role in limiting the collateral tissue damage caused by both the inflammatory and oxidative response to injury. Originally viewed as a hormone which stimulates only pro-erythroblasts, EPO is now known to be produced in many tissues, after a temporal delay, to promote healing following injury or infection and to stimulate the production and recruitment of stem cells (Hand and Brines, 2011)

The high doses of rhEPO required for tissue protection and inhibition of apoptosis in non-erythroblast cells are associated with very real risks of significant clinical complications. In fact, even moderate doses of rhEPO or other ESAs (including Darbopoetin) administered chronically have been associated with adverse consequences in patients. Large scale clinical studies involving higher doses of ESAs administered as treatment for anemia or to improve quality of life have shown that as a class, ESAs reduce overall survival and/or increase the risk of tumor progression and recurrence in patients with breast, non-small cell lung, head and neck, lymphoid, and cervical cancer. Multiple studies employing both animal models and patients indicate that ESAs augment thrombopoiesis, as well as platelet and endothelial cell activation (Haiden et al., 2005; Kato et al., 2010; Stasko et al., 2007; Stohlawetz et al., 2000; Wolf et al., 1997a, 1997b).

The resulting thrombotic complications of ESAs are dose-related (Bohlius et al., 2009). The most serious complications associated with ESAs occurred in a recent trial of rhEPO, given at 40,000 IU/day for 3 days after stroke, in which rhEPO-treated patients had a higher rate of intracranial hemorrhage and a higher mortality rate (Ehrenreich et al., 2009).

We have recently demonstrated that the hematopoietic and tissue- protective effects of EPO can be separated (Leist et al., 2004) We have also identified a novel cell surface receptor, the Tissue-protective Receptor (TPR), comprised of one classical EPO receptor (EpoR) and a pair of CD131 (beta common) receptor subunits, that may mediate the non-hematopoietic effects of EPO (Brines et al., 2004). The molecular interaction of EPO with the homodimer erythropoietic receptor complex ((EpoR)₂) has been extensively studied and critical regions within EPO that interact with (EpoR)₂ have been identified (**Figure 1**). These include portions of helices A and C (site 2) as well as helix D and the loop connecting helices A and B (site 1).



We have developed receptor-selective agents that bind only to TPR and are devoid of EPO-related side effects in animal models. Unlike EPO itself, rhEPO derivatives that bind only to TPR do not increase pro-coagulant activity or effect platelet production [27]. We have now used and published three distinct and successful approaches: (1) to make mutations in rhEPO; (2) to chemically modify rhEPO and (3) to make peptide derivatives of EPO that mimic its 3D structure (Brines et al., 2008).

Body:

We and our collaborators have now shown that a short EPO-mimetic peptide, Helix-B surface peptide (HBSP), improves survival in a murine model of acute radiation induced Bone Marrow Failure (**Table 1**).

Table 1:

Grp	RAD Dose	Treatment schedule (days injected)	No. of Survivors/Total	30 day Survival (%)	MST of Decedents (days)	30d Survival (p value vs. Vehicle)	MST (p value vs. Vehicle)	Overall Survival Time (p value vs. Vehicle)
HBSP	7.96	d1-30	9/20	45	15.7	0.002	0.387	0.002
	8.31	d1-30	4/20	20	17.4			
PBS	7.96	d1-30	2/20	10	16.7			
	8.31	d1-30	1/20	5	15.1			
Radiation Dose: 7.96 Gy total body irradiation (LD 70/30) on day 0, or 8.31 Gy total body irradiation (LD 90/30) on day 0 HBSP dose: 25 µg/kg, administered subcutaneously starting 24 hours after radiation exposure and was given daily for 29 days.								

However, mostly due to an overwhelming death rate in the control groups (PBS treated), these results have not consistently observed in follow up studies (**Table 2 and 3**).

Table 2:

Group Description (RAD dose)	Treatment schedule (days injected)	No. of Survivors/Total	30 day Survival (%)	MST of Decedents (days)	30d Survival (p value vs. Vehicle)	MST (p value vs. Vehicle)	Overall Survival Time (p value vs. Vehicle)
HBSP	d1	18/30	60	22.4	1.0	0.764	>0.1
	d1-10	7/30	30	19.8			
PBS	d1	15/30	50	20.9			
Radiation Dose: 7.96 Gy total body irradiation (LD70/30) on day 0. HBSP dose: 25 µg/kg, administered subcutaneously starting 24 hours after radiation exposure and was given daily for 29 days.							

Table 3:

Group Description (RAD dose)	Treatment schedule (days injected)	No. of Survivors/Total	30 day Survival (%)	MST of Decedents (days)	30d Survival (p value vs. Vehicle)	MST (p value vs. Vehicle)	Overall Survival Time (p value vs. Vehicle)
HBSP	d1	18/30	60	22.4	1.0	0.764	>0.1
	d1-10	7/30	30	19.8			
PBS	d1	15/30	50	20.9			
	d1-10	17/30	57	20.4			
Radiation Dose: 7.96 Gy total body irradiation (LD70/30) on day 0. HBSP dose: 25 µg/kg, administered subcutaneously starting 24 hours after radiation exposure and was given daily for 29 days.							

Preliminary Safety Studies in small mammals and humans on HBSP (summarized in Table 4)

Hematopoietic Effects: No consistent increase or decrease was seen in hematocrit, hemoglobin, red blood cell count, reticulocyte count, platelet count, or levels of ferritin or transferrin in 10-day twice-daily dosing in rats and rabbits at up to 6,000 and 3,000 ug/kg/dose, respectively, the highest doses tested, nor in 28 day, twice-daily dosing in rats and rabbits up to 600 and 300 ug/kg/dose, respectively, the highest doses tested. HBSP showed no erythropoietic or hematopoietic activity in multiple animal toxicology studies in the rat and rabbit, nor in any long-term efficacy study with chronic dosing. No evidence of the formation of antibodies that neutralize endogenous EPO activity was observed in any study.

Toxicity Studies: In a standard safety pharmacology assessment program, respiratory and central nervous system function in rats showed no adverse effects at up to the highest dose tested of 6,000 ug/kg. No adverse cardiovascular effects were seen in telemeterized rabbits at up to the highest dose tested of 3,000 ug/kg. In addition, no inhibition of the hERG potassium channel was evident at the highest concentration tested, 30 micromolar (38 mg/ml), in an in-vitro assay using hERG-transfected human embryonic kidney cells. No observable adverse effects were seen at doses of up to 3,000 and 6,000 mg/kg, when administered twice daily repeat-dose studies of ten days' duration in rabbits and rats, respectively. Bacterial reverse mutation and eukaryotic chromosomal aberration studies showed no propensity for HBSP to induce mutagenic or genotoxic effects, up to the highest concentrations tested, 5,000 mg/plate or 5000 mg/mL, respectively.

Clinical studies: Three phase 1 studies have been performed in normal subjects in doses up to 2000 ug in single doses and in a multiple dose regiment up to 1000 ug every 12h for 9 doses. No adverse events were identified in these studies.

Table 4. Phase 1 Clinical Studies of HBSP

Study Type, Number of subjects (Dose Levels)	Parameters Measured	Results
Single ascending IV dose in 46 healthy volunteers (70, 210, 500, 700, 1000, 2000 µg) (5 active, 2 placebo per dose level of most groups)	All subjects: safety, tolerability, PK, CDR cognitive functional battery.	Safe and well tolerated. No increase in RBC of platelets. No upregulation of P-selectin.
Multiple ascending IV dose in 14 normal volunteers (500 or 1000 µg every 12h for 9 doses)	Safety, tolerability, PK.	Safe and well tolerated. No increase in RBC of platelets. No upregulation of P-selectin.
Single dose of 1400 µg IV-SC cross-over study in patients with impaired renal function (additional arm of above study)	Safety, tolerability, cytokine levels.	Safe and well tolerated. No increase in RBC of platelets. No upregulation of P-selectin or changes in cytokine levels.

Objective 1: Determine if HBSP inhibits apoptosis in murine and human hematopoietic stem cells.

Nonhematopoietic derivatives of EPO, are known to inhibit apoptosis in a wide range of tissue and cell types including endothelial progenitor cells, neurons, renal cells, retinal ganglion cells and cardiomyocytes. We studied the hypothesis that HBSP inhibits radiation induced apoptosis in the following hematopoietic cell lines: HL-60, NB-4 cells, 32Dc13 and EML cell line.

Experimental design and methods:

HL-60, a cell line derived from peripheral blood leukocytes of a 36-year-old Caucasian female with acute promyelocytic cells, were obtained from ATCC and grown in RPMI media containing 10% Fetal Bovine Serum (FBS). New cultures were started at 2×10^5 viable cells/mL and subculture at 1×10^6 cells/mL. Media was changed every 2 or 3 days. Care was taken to insure that the cells were not at the log phase of growth when all experiments were performed.

NB-4, a promyleocytic cell line, was grown in RPMI 1640 containing 2mM L-glutamine with 10% FBS. This cell line grows in suspension and was maintained at a density between 0.5×10^6 cells/ml and 1×10^6 cells/ml .

32Dc13, a murine hematopoietic precursor cell line, was grown in DMEM with 10% FBS. Cells were plated at low density and adjusted two to three times per day after the addition of fresh media. Cells were differentiated as in (Sood et al., 1999).

EML Cell line (ATCC CRL-11691), a bone marrow cell line obtained by immortalizing bone marrow cells from male BDF1 mice with a retrovirus, was grown in DMEM with 4mM L-glutamine, 1.5 g/l sodium bicarbonate, 200 ng/ml mouse stem cell factor, 20% fetal bovine serum. Cultures were maintained at the recommended cell concentrations between 4×10^5 and 2×10^6 viable cells/mL and media was changed every 2 to 3 days.

Cells of each cell type were plated a density of 100,000 cells/well then treated with HBSP at the following concentrations 2ng/ml, 4ng/ml, 8ng/ml, 20 ng/ml, 40 ng/ml, 80 ng/ml, 160 ng/ml and 200ng/ml at the following time points: 48 hours prior to induction of apoptosis, 24 hours prior to the induction of apoptosis, at the time of induction of apoptosis, 2 hours after the induction of apoptosis, 6 hours after the induction of apoptosis and 24 hours after the induction of apoptosis. All experiments were completed a minimum of three times and were performed in triplicate.

Apoptosis was induced by irradiating cells with γ -rays (0.1–5 Gy) using a $^{137}\text{Cesium}$ source delivering a dose rate of 0.82 Gy/min. Cells were then assayed for apoptosis at 48 h after the induction of apoptosis by staining for Annexin V. In brief, cells were harvested, washed and then resuspended in 1X binding buffer at 1.5×10^6 cells/ml. Next, 5 μL of fluorochrome-conjugated Annexin V was added to 100 μL of the cell suspension. Incubated for 10 min at room temperature. Cells were then washed 3 times and stained with propidium iodide staining solution (Nexcelom). Cells were then stored at 4C in the dark until they were analyzed using a Cellometer 2000 cell viability counter (Nexcelom).

Results:

No statistically significant inhibition of apoptosis was detected at any of the compound doses tested in any of the cell lines tested. Additional studies were done looking at the effect of HBSP on the apoptosis induced by staurosporine.

apoptosis in the HL-60 cell line using staurosporine. other methods of induction of apoptosis including NMDA and. No statistically significant anti-apoptotic effects were seen under any of the tested conditions.

Objective 2: Isolate and characterize the HBSP receptor from murine and human hematopoietic stem cells using affinity chromatography and mass spectrometry.

Experimental design and methods:

Membrane preparations were made from HL-60, NB-4, 32Dc13 and EML cells attempts were made to co-immunoprecipitate the CD131 molecule with EPOR in these two cells lines. Specifically, cells were grown to a concentration of 5×10^6 , pelleted, washed once with sterile PBS and then the pellet was solubilized with lysis buffer (1% Triton X100 (Sigma), 150 mmol/L NaCl 50 mmol/L Tris pH 8.2, with 10 mg/mL leupeptin, 0.2 U/mL aprotinin, 10 mg/mL leupeptin, 2 mmol/L phenylmethylsulfonyl fluoride for 15 minutes on ice in a 4 C cold room. Lysates were then centrifuged at 14,000 rpm using a refrigerated Eppendorf Centrifuge. Supernatants were then pre-cleared with mouse sera and sepharose beads for 1 hour and then incubated with either of the following antibodies anti-EPOR (M-20, sc-697, Santa Cruz, CA, epitope mapped within a C-terminal cytoplasmic domain of EpoR) or CD131 (sc-678, IL-3/IL-5/GM-CSFRbeta, K-19, Santa Cruz, CA) or preimmune control antibodies for a minimum of 8 hours at 4C. Protein A Sepharose beads were then added and incubated with the lysates for 2 hours at 4C with shaking. Beads were then washed three times using PBS and divided into two aliquots. Half of the beads were boiled in SDS gel loading buffer [100 mM Tris-Cl (pH 6.8), 4% (w/v) sodium dodecyl sulfate, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM beta mercaptoethanol and the other half were boiled in SDS gel loading buffer without mercaptoethanol. Samples were run on 7.5% gels (Biorad precast gels) and bound proteins were electrically transferred to Immobilon (Millipore) and subsequently immunoblotted to detect the presence of either CD131 (sc-678) or EPOR (sc-697). A positive control consisted of similar extracts made from the erythroleukemic cell line, K562.

Results: There was no evidence that CD131 was expressed in association with EPOR in any of the cell lines tested. HBSP has radio protective activity in vivo in a murine model of bone marrow failure. However, at this point, the cellular or molecular mechanisms of this protection is unknown. Experiments to date, have been aimed at uncovering an anti-apoptotic effect in stem cell precursors. Future work will be directed toward the study of the hypothesis that like EPO, HBSP may prevent or reverse DNA damage (Rjiba-Touati et al., 2012, 2013) and investigating the effects of other molecules that stimulate HIF-1alpha in bone marrow failure (Forristal et al., 2013).

KEY RESEARCH ACCOMPLISHMENTS

During this grant period, we tested the following hypothesizes:

- 1) The Epo-mimetic peptide will decrease mortality associated with Bone Marrow Failure secondary to toxic radiation exposure.
- 2) The Epo-mimetic peptide directly inhibits apoptosis of hematopoietic stem cells
- 3) The Epo-mimetic peptide binds to the tissue protective receptor complex composed of EPOR and CD131

We found:

- Epo-mimetic peptide decreases mortality associated with bone marrow failure
- Epo-mimetic peptide does not inhibit apoptosis in the hematopoietic stem cell lines tested.
- Epo-mimetic peptide does not bind to tissue protective receptor complex composed of EPOR and CD131 in hematopoietic stem cell lines tested.

REPORTABLE OUTCOMES:

Manuscript in preparation for submission to Journal of Experimental Hematology:

C. Hand and G. Gipson. Radioprotective effects of Erythropoietin, 2013.

CONCLUSION:

During this grant period, We found:

- Epo-mimetic peptide decreases mortality associated with bone marrow failure
- Epo-mimetic peptide does not inhibit apoptosis in the hematopoietic stem cell lines tested.
- Epo-mimetic peptide does not bind to tissue protective receptor complex composed of EPOR and CD131 in hematopoietic stem cell lines tested.

HBSP has radio protective activity in vivo in a murine model of bone marrow failure. However, at this point, the cellular or molecular mechanisms of this protection is unknown. Experiments to date, have been aimed at uncovering an anti-apoptotic effect in stem cell precursors. Future work will be directed toward the study of the hypothesis that like EPO, HBSP may prevent or reverse DNA damage (Rjiba-Touati et al., 2012, 2013) and investigating the effects of other molecules that stimulate HIF-1alpha in bone marrow failure (Forristal et al., 2013).

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